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Original Article

Thiadiazole carboxylic acid moiety of tiadinil, SV-03, induces systemic acquired resistance in tobacco without salicylic acid accumulation

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Systemic acquired resistance (SAR) is a potent innate immunity system in plants that is effective against a broad range of pathogens and induced through the salicylic acid (SA)-mediated pathway. Here we have characterized the SAR induction activity of 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (SV-03) identified as a metabolite of tiadinil in rice. Soil drench application of SV-03 induces a broad range of disease resistance and *PR* gene expression in tobacco. Further analyses using NahG transgenic tobacco plants indicate that SV-03-induced resistance enhancement does not require SA. Therefore, it is suggested that SV-03 induced SAR by triggering signaling at the same level as or downstream of SA accumulation. © Pesticide Science Society of Japan

Keywords: systemic acquired resistance (SAR), salicylic acid, tobacco, disease resistance.

Introduction

A local hypersensitive response to pathogens induces systemic acquired resistance (SAR), a plant defense response, which plays an important role in protecting plants from further attacks by various kinds of pathogens.[1,2] The induction mechanism of SAR has been well-characterized in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana*, and a set of pathogenesis-related (*PR*) genes has been identified as SAR marker genes.[3–6] Several chemicals capable of inducing SAR have been reported; salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), benzo(1,2,3)thiadiazole-7-carbothionic acid *S*-methyl ester (BTH), 3-allyloxy-1,2-benzisothiazole-1,1-dioxide (probenazole; PBZ), *N*-cyanomethyl-2-chloroisonicotinamide (NCI), and 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid (CMPA) (Fig. 1).[4,6–14] These chemicals exhibit several essential criteria of SAR inducers: they induce disease resistance against a broad range of pathogens; their effects are not due to their antibiotic activities; and they induce an SAR molecular marker, *PR* gene expression.[4,15] Some of these chemicals have been used practically in the field to control diseases such as rice blast. Despite extensive studies on the

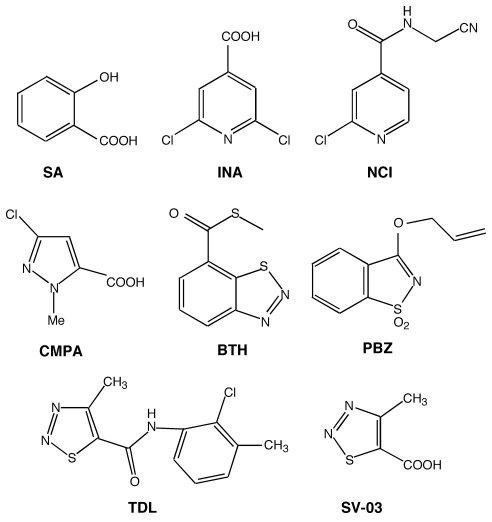

Fig. 1. Chemical structures of SAR inducers and SV-03. SA, salicylic acid; INA, 2,6-dichloroisonicotinic acid; NCI, *N*-cyanomethyl-2-chloroisonicotinamide; CMPA, 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; PBZ, probenazole; TDL, tiadinil.

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induction mechanism of SAR over the years, many parts of the SAR signaling pathway remain to be clarified; for example, no target of these chemicals has been determined thus far.

Tiadinil (TDL), *N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide, has been developed by Nihon Nohyaku Co., Ltd. and is used practically to control rice blast disease. Studies using tobacco plants revealed that TDL is an SAR activator.[16] Recently, it was reported that 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (SV-03), a metabolite of TDL, exhibited anti-rice blast activity, suggesting that SV-03 is presumably an active component of TDL.[17] As tobacco is a suitable plant model for the evaluation of SAR inducers, we assessed the possibility that SV-03 induces disease resistance in this plant. Here we show that SV-03 exhibits all the criteria of SAR inducers in tobacco, and can induce a broad range of disease resistance by stimulating the SAR signaling pathway downstream of SA.

Materials and Methods

1. Chemicals

SV-03 was a gift from Nihon Nohyaku Co., Ltd.

2. Plant materials and treatment

Nicotiana tabacum cv. Xanthi nc was grown in sterilized potting soil (Kureha, Japan) in pots (6 cm diameter×9 cm) inside a growth chamber under a 16 : 8 light : dark regimen, at 22°C, with 60% humidity. Pretreatment with various concentrations of chemicals or water was performed by soil drenching of 5-week-old plants with 5–6 developed leaves.

3. Tobacco pathogen infection assays

In tobacco mosaic virus (TMV) infection assay, 5 days after treatment with SV-03, a challenge inoculation with TMV was performed. The three developed leaves of treated plants were dusted with carborundum (Mesh 400; Maruto Co., Japan) and mechanically inoculated with TMV suspended in 10 mM phosphate buffer (pH 7). The inoculated plants were incubated at 22°C and lesions were measured 5 days later.

A challenge inoculation with *Pseudomonas syringae* pv. *tabaci* (*Pst*), a pathogen of tobacco wildfire disease, was performed 5 days after treatment with SV-03. *Pst* was cultured in nutrient broth for 1 day at 28°C, and a bacterial suspension was prepared in 10 mM $MgCl_2$ (2×10^5 colony-forming units (CFU)/ml). Challenge inoculation was performed by infiltration of the bacterial suspension by gently pressing a 1 ml syringe without a needle.[18] Leaf disks (6 mm diameter) were taken from the infiltrated part of the leaves at 4 days post inoculation and three disks from each plant were combined and homogenized in 200 μl of 10 mM $MgCl_2$. The population of *Pst* was estimated by growth on nutrient broth agar plates after dilution.

4. Antimicrobial activity

P. syringae pv. *tabaci* was precultured for 1 day and the same aliquots (40 μl) were inoculated to new nutrient broth cultures (2 ml each) containing SV-03. Culture was performed with shaking for 8 hr at 28°C and the OD_{600} was measured.

5. RNA analysis

Tobacco plants were treated with various concentrations of SV-03 or water by the soil drench method, and the leaves were harvested at 5 days after application. Total RNA was extracted from frozen leaf samples of the plants using Sepasol-RNA I super reagent (Nacalai Tesque Co. Ltd., Japan) following the manufacturer's instructions. DNA fragments of the coding regions for tobacco *PR* genes[4] were amplified by polymerase chain reaction (PCR) from cDNA prepared from SA-treated tobacco.[9,10] The amplified DNA fragments were cloned into plasmid pCR2.1 (Invitrogen, CA, U.S.A.) and the nucleotide sequences were confirmed. ^{32}P-labeled cDNA probes were synthesized by random priming of these fragments of acidic *PR-1a*, *PR-2*, *PR-5* and basic *PR-1* genes. Total RNA samples were subjected to 1.2% agarose-1.1% formaldehyde gel electrophoresis and transferred to a nylon membrane (Hybond N+, Amersham, Buckinghamshire, U.K.). After the transfer, RNA was cross-linked to the membrane using a u.v. linker (GS GENE LINKER, Bio-Rad, Hercules, CA, U.S.A.). Prehybridization and hybridization were performed at 68°C for 1 hr or longer and 8 hr or longer, respectively. The membrane was washed twice with 2×SSC containing 0.1% SDS for 30 min at 68°C and then washed twice with 0.1×SSC containing 0.1% SDS for 15 min at 68°C. Detection was performed with a BAS2500 image analyzer (Fuji Photo Film Co., Ltd.).

6. Extraction and analysis of SA

Plants were treated with SV-03 or water and samples were harvested 5 days after application. Harvested samples (4–6 g) were homogenized and extracted with 20 ml of 90% methanol and then with 20 ml of 100% methanol. These two extracts were combined and 2 ml of this extract was dried at 35–40°C. The dried residue was extracted with 4 ml of water at 80°C for 10 min and the same aliquot (1 ml) was used for free and total SA analysis. One aliquot was extracted with 2.5 ml ethyl acetate–cyclohexane (1 : 1) following the addition of 50 μl of conc. HCl, and the upper layer was dried and dissolved in 1 ml of 20% methanol in 20 mM sodium acetate buffer (pH 5). This was applied to HPLC analysis as a free SA sample. To the other aliquot of water extract 1 ml of β-glucosidase solution (3 unit/ml) was added and incubated for 6 hr at 37°C. This was extracted with 2.5 ml ethyl acetate–cyclohexane (1 : 1) following the addition of 50 μl of conc. HCl and the HPLC sample was prepared as well as for free SA. SA analysis was performed by 8000 series HPLC system (Japan Spectroscopic Co., Ltd.) using a TSK-gel ODS 80 column (4.6× 150 mm, Toso Co.) running with 20% methanol in 20 mM sodium acetate buffer (pH 5) at a flow rate of 1 ml/min. SA was detected and quantified fluorometrically (295 nm excitation and 370 nm emission).

Results

1. Induction of a broad range of disease resistance in tobacco by SV-03

The ability of SV-03 to enhance resistance to pathogen infection in tobacco was assessed using TMV. *Nicotiana tabacum* cv. Xanthi nc possesses the *N* gene, which confers resistance to tobacco mosaic virus (TMV)[19]; consequently, its defense response to TMV infection results in a necrotic lesion. SA and SAR activators enhance this resistance and reduce the size of lesions.[2,4,9,10,13,20] SV-03 treatment was performed using soil drenching, with challenge inoculation performed 5 days later. The lesions appearing on the leaves of SV-03-treated plants were smaller than those of water-treated control plants, indicating that SV-03 enhanced *N* gene-mediated resistance against TMV (Fig. 2).

Next, we assessed the effect of SV-03 on the interaction between tobacco plants and a virulent bacterial pathogen, *Pseudomonas syringae* pv. *tabaci* (*Pst*). *N. tabacum* cv. Xanthi nc does not have a resistance gene specific to *Pst* and the relationship between this plant and *Pst* is compatible. Susceptibility was estimated by measuring bacterial growth in leaf tissues after challenge inoculation. Treatment with SV-03 at 1 or 2.5 mg/pot by soil drenching reduced bacterial growth in the infected tissues relative to the water-treated control plants, resulting in a reduction in disease symptoms (Fig. 3). SV-03 did not show any direct anti-microbial activity in liquid culture at concentrations of up to 0.25 mg/ml (Table 1). This indicates that SV-03 induces resistance to *Pst* in tobacco plants. Considering all the results of inoculation tests, we conclude that

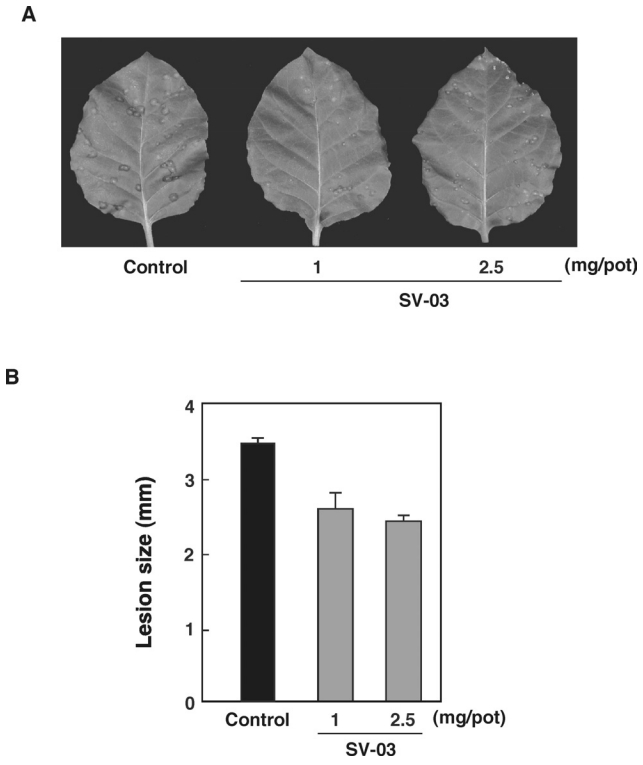

Fig. 2. Induction of resistance in tobacco against TMV by SV-03. Tobacco plants were treated with 1 or 2.5 mg/pot TDL or water by soil drench method, 5 days prior to TMV inoculation. (A) Photograph was taken 5 days post inoculation. (B) Average size of lesions was measured 5 days after TMV inoculation. Each experiment was performed with three plants and three leaves of each were inoculated with TMV. Values are shown as the means±SD. The experiment was repeated three times with similar results.

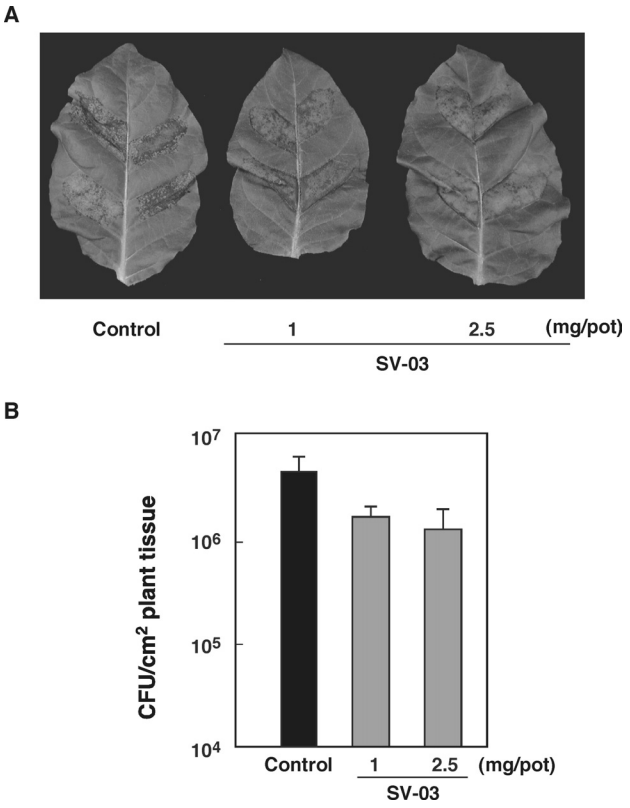

Fig. 3. Induction of resistance against bacterial pathogen by SV-03. Five-week-old tobacco plants were treated with water (control) or 1, 2.5 mg/pot SV-03 by the soil drench method 5 days prior to inoculation with pathogen (*Pst*). (A) Symptoms of tobacco wildfire disease on tobacco leaves. Photograph was taken 7 days post inoculation. (B) Growth of *Pst* in tobacco leaf tissues. The population of *Pst* in the sample was estimated 4 days after inoculation by growth on nutrient broth agar plates. Each experiment was performed with three plants and two samples were prepared from each plant by homogenizing leaf disks in 10 mM $MgCl_2$. Values are shown as the means±SD. The experiment was repeated three times with similar results.

Table 1. Effect of SV-03 on growth of *P. syrinage* pv. *tabaci*

SV-03 concentration (mg/ml)	Bacterial growth (% Cont.)
0.25	101
0.10	107
0.025	105
0.010	109

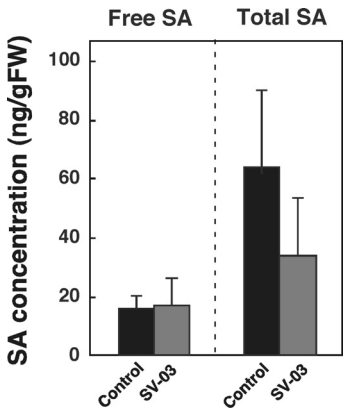

Fig. 4. Accumulation of free and total salicylic acid in wild-type plants treated with SV-03. Leaves were harvested 6 days after treatment with SV-03, and the free and total SA (free SA+SAG) levels were quantified by HPLC.

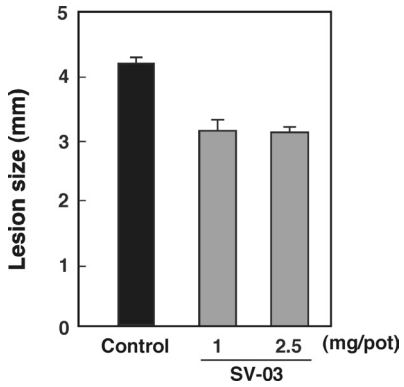

Fig. 5. Induction of resistance in NahG transgenic tobacco against TMV by SV-03. Tobacco plants were treated with 1 or 2.5 mg/pot SV-03 or water (control) by the soil drench method, 5 days prior to TMV inoculation. Average size of lesions was measured 5 days after TMV inoculation. Each experiment was performed with three plants and three leaves of each were inoculated with TMV. Values are shown as the means±SD. The experiment was repeated three times with similar results.

SV-03, similarly to TDL, induces SAR-like disease resistance to a broad range of pathogens in tobacco.

2. Induction of disease resistance in tobacco without accumulation of SA by SV-03

Biologically induced SAR accompanies SA accumulation even in healthy leaves.[2)] To examine whether SV-03 induces SA accumulation in tobacco, the endogenous levels of free and total SA (free SA plus salicylic acid glucoside, SAG) were measured in SV-03- and water-treated tobacco plants 6 days after treatment. The levels of free and total SA detected in the SV-03-treated plants were not significantly different from those observed in the water-treated controls (Fig. 4). This result indicates that SV-03 does not induce SA accumulation in tobacco plants.

To determine whether SV-03 requires SA for disease resistance induction, we examined the effect of SV-03 on NahG transgenic tobacco plants unable to accumulate SA due to the expression of salicylate hydroxylase, an SA-degrading enzyme.[5,6)] The average lesion size in SV-03-treated NahG plants was smaller than that of water-treated control plants, whereas NahG transgenic plants exhibit larger lesions in response to TMV infection compared to those in wild-type plants (Figs. 2 and 5). In addition, NahG plants treated with SV-03 also exhibited statistically significant resistance against *Pst* (Fig. 6). These results indicate that SV-03 does not require SA to induce SAR-like disease resistance.

3. Induction of PR gene expression by SV-03

Some PR proteins are coordinately expressed in tobacco leaves during the induction and maintenance of SAR and these are also expressed during SAR induced by chemical plant activators such as SA and BTH.[2,4,9,13)] The expression of acidic *PR-1* (*PR-1a*), encoding one of these PR proteins, is a useful molecular marker for SAR and its expression in SV-03-treated plants was examined. Northern blot analysis indicated that the transcript for acidic *PR-1* accumulated in tobacco leaves treated with SV-03 in a dose-dependent manner (Fig. 7). The transcripts for acidic *PR-2* (β-1,3-glucanase) and *PR-5* (thaumatin-like) also accumulated in the leaves of SV-03-treated plants dose-dependently (Fig. 7). By contrast, none of these transcripts could be detected in the leaves of water-treated control plants (Fig. 7). The ability of SV-03 to induce SAR marker gene expression and enhance disease resistance in the absence of antibacterial activity confirms that SV-03 can activate SAR in tobacco plants. Treating NahG plants with SV-03 also induced the expression of *PR-1*, *PR-2* and

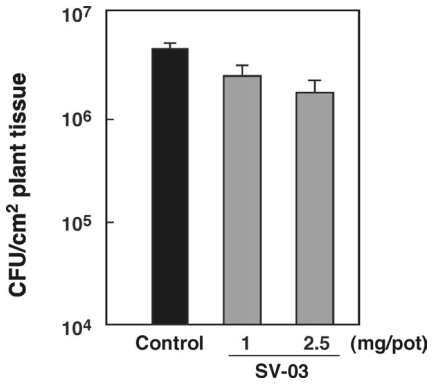

Fig. 6. Induction of resistance in NahG transgenic tobacco against bacterial pathogen by SV-03. Five-week-old tobacco plants were treated with water (control) or 1, 2.5 mg/pot SV-03 by the soil drench method 5 days prior to inoculation with pathogen (*Pst*). The population of *Pst* in tobacco leaf tissues was estimated 4 days after inoculation. Each experiment was performed with three plants and two samples were prepared from each plant by homogenizing leaf disks in 10 mM $MgCl_2$. Values are shown as the means±SD. The experiment was repeated three times with similar results.

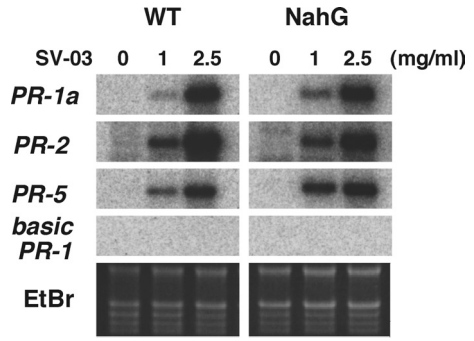

Fig. 7. Induction of SAR marker gene expression in tobacco by SV-03. Wild-type and NahG transgenic tobacco plants were treated with SV-03 (1 or 2.5 mg/pot) or water (control) by the soil drench method. Leaves were collected 5 days after treatment and used for Northern blot analysis following RNA extraction. Each lane was loaded with 4 μg total RNA. Equal loading was confirmed by ethidium bromide staining.

PR-5 genes similarly to in wild-type plants (Fig. 7), indicating that SV-03 is effective in inducing defense gene expression in an SA-independent manner. The data obtained using NahG plants strongly suggest that SV-03 does not require SA biosynthesis to develop SAR.

On the other hand, in tobacco, wounding stress activates jasmonic acid-mediated signaling pathway and induces systemic expression of basic *PR* genes. To determine whether SV-03 activates this defense response in tobacco, we analyzed its ability to induce basic *PR-1* gene expression in wild-type and NahG tobacco plants. Northern blot analysis indicated that transcripts for basic *PR-1* gene did not accumulate in the leaves of SV-03-treated plants or water-treated control plants (Fig. 7), indicating that SV-03 induces the SAR signaling pathway but not the jasmonic acid-mediated signaling pathway.

Discussion

In this paper, we demonstrate that SV-03 induces a broad range of disease resistance in tobacco. SV-03 enhances resistance against the viral pathogen TMV and the bacterial pathogen *Pst*, but does not exhibit antibacterial activity. During resistance induction, SV-03 induces *PR* gene expression in tobacco. Therefore, SV-03 fulfills all the criteria of an SAR inducer in tobacco. Furthermore, measurement of SA accumulation in wild-type plants and the use of NahG transgenic tobacco plants revealed that SV-03-mediated resistance enhancement in tobacco does not require SA; this suggests that SV-03 induces disease resistance by triggering the signaling pathway at the same level as or downstream of SA, as do BTH, INA, NCI and CMPA.

Recently, it was reported that SV-03 accumulates in tissues of TDL-treated rice plants and possesses anti-rice blast activity. The data shown here demonstrated that SV-03 has SAR-inducing activity in tobacco. Although it is still unknown whether TDL is converted to SV-03 in tobacco plants, it is possible that both TDL and SV-03 contribute to SAR induction and anti-rice blast activity. Therefore, for the present, it cannot be determined whether the conversion of TDL to SV-03 is necessary for the induction of disease resistance.

Several heterocyclic chemicals, such as PBZ, BTH, INA, NCI and CMPA, have been known to induce SAR with no or low direct antimicrobial activity *in vitro*. This study revealed that SV-03 is a novel class of SAR inducer containing thiadiazole skeleton in its structure. The data presented here indicate that the mode of action of SV-03 is similar to those of BTH, INA, NCI and CMPA, however, it remains to be clarified whether these chemicals act on the same target protein. In *Arabidopsis*, NPR1 is a key regulator of SAR and functions downstream of SA.[21–23] SV-03 exhibits all the necessary criteria for an SAR inducer, as reported here, and it would be interesting to examine whether SV-03 requires NPR1 for SAR induction. Furthermore, various *Arabidopsis* mutants have defects in the SAR signaling pathway, including proteins interacting with NPR1. Therefore, the detailed mechanism of SV-03-induced resistance will likely be clarified using *Arabidopsis*, and this is currently under investigation.

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